CERTIFICATE OF MAILING

I hereby certify that this paper and every paper referred to therein as being enclosed is being deposited with the U.S. Postal Service as first class mail, postage prepaid, in an envelope addressed to: Commissioner of Patents & Trademarks, Washington, DC 20231, on August 31, 2000 (Date of Deposit)

8/31/00 A. DiLullo
Date Name

0630/0D532

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

NEUHOLD et al.

Serial No.: 08/994,689 Group Art Unit: 1633

Filed: December 19, 1997 Examiner: M. Wilson

For: TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE

SECOND DECLARATION OF LISA A. NEUHOLD, Ph.D.,
UNDER 37 C.F.R. § 1.132

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

LISA A. NEUHOLD, Ph.D., declares and states that:

1. I am a co-inventor of the above-identified patent application.

2. I presently hold a position as Senior Research Scientist I, Molecular Genetics, for Wyeth-Ayerst Research, Andover, Massachusetts, which is a division of American Home Products Corporation, assignee of this application and the invention.

3. My qualifications as a scientist are set forth on the copy of my *curriculum vitae* which is attached as Exhibit A to the Declaration of Lisa A. Neuhold, Ph.D. Under 37 C.F.R. § 1.132 filed April 6, 1999.

4. I have read and am familiar with the Office Action dated March 1, 2000. I understand that claims 1, 2, 4-9, 11, 12, 15-17, and 22-28 have been rejected for lack of enablement. It is my understanding that the Examiner believes that the specification does not teach how to make transgenics in any other species other than mice and that the teachings of this application fail to provide a correlation between the effects of mutations in MMP, specifically MMP13, and cartilage-related diseases. The following data, which have been obtained in accordance with the teachings of this application, further demonstrate that the transgenic animal model of the invention is an effective animal model that mimics human osteoarthritis, also called degenerative joint disease, and related pathological syndromes; that regulated expression of an extracellular matrix degrading enzyme under control of inducible promoters and chondrocyte specific promoters functional in a number of different mammalian cells yield transgenic animals with the claimed phenotype after essential stages of development.

5. I participated in the interview at the Patent and Trademark Office on May 17, 2000, during which we discussed issues of enablement of creation of transgenic animals in accordance with this invention, including the criteria for an animal model of osteoarthritis, features of various inducible expression systems, generality of tissue-specific expression systems, generality of extracellular matrix degrading enzymes for damaging the joint extracellular matrix to induce the phenotypic changes characteristic of osteoarthritis, the relative ease in preparing

transgenic animals and thus of copying the invention without directly copying the exemplified embodiments, and that the phenotype of two lines of transgenic animals we have created in accordance with the invention correspond to the phenotypic characteristics of osteoarthritis.

6. As discussed during the interview, a number of expression systems are available that permit inducible expression in transgenic animals. These include the ecdysone inducible system, the RU 486 inducible system, reversed tetracycline inducible system (in which expression occurs in the presence of the tetracycline compound), and the tetracycline repressible system as exemplified in the application (in which expression occurs in the absence of the tetracycline compound). These systems are summarized in the context of the present invention (*i.e.*, expression of MMP-13 as the extracellular matrix degrading enzyme under control of the inducible promoter, and expression of the inducible promoter under tissue specific control of the collagen II promoter) in Tab 1. Thus, a researcher who wished to make a transgenic animal of the invention would have a any number of of inducible expression systems to choose from. Furthermore, as discussed at the interview, these inducible systems are fungible. Nothing about the particular inducible expression system, other than that it is inducible, makes a difference in generation of the transgenic animals.

7. We also discussed tissue-specific expression. The examiner seems to be of the opinion that only a collagenase II promoter will permit tissue-specific expression. While expression in chondrocytes under control of a chondrocyte tissue-specific promoter ensures that the effects of the extracellular matrix degrading enzyme are localized to joints, and thus that the phenotype the animals develop represents a localized joint pathology, the identity of the tissue-

specific promoter is not important. The type II collagen promoter is a well known and thus logical, but by no means essential choice. Other chondrocyte-specific promoters can be substituted for it. As set forth in the memo attached at Tab 2, the CD-RAP/MIA gene promoter is one such substitute for the type II collagen promoter. Other chondrocyte-specific genes, whether known now or discovered in the future, similarly can be used for tissue-specific expression of the transcriptional regulator polypeptide, as any ordinary skilled molecular biologist would know. This reflects the generally understood principle that tissue-specific expression control sequences are well suited for expression of transgenes as well as the endogenous gene (see, for example, the paper attached to the Preliminary Amendment filed by hand, Tab E). Nothing about the transgenic animals that we have generated, or based on the examiner's contentions in the Office Action, contradicts this conclusion.

8. We discussed whether the transgenic animals must employ the constitutively active matrix metalloproteinase 13 (MMP-13), or if other matrix metalloproteinases can be used. We discussed that collagen degradation, and particularly collagen II degradation, is a feature of osteoarthritis. In addition to MMP-13, MMPs 1 and 8 also specifically degrade collagen II, and thus will certainly be effective in transgenic animal models of this disease. MMP-1 has been expressed in transgenic animal models (though not in a chondrocyte tissue-specific manner), as summarized on the slide attached at Tab 3, which was presented at the interview. Furthermore, although a constitutively active MMP-13 enzyme was employed in the examples, in other transgenic systems wild-type MMPs were also effective. MMPs are typically activated by endogenous proteases. Furthermore, as set forth in Table 1 of

the patent application (at pages 2-3), there are a large number of other matrix degrading enzymes capable of destroying the extracellular matrix components of joint tissue. Thus, any enzymatically active extracellular matrix degrading enzyme, and particularly those that degrade type II collagen, can be used to generate transgenic animals that demonstrate phenotypic characteristics of osteoarthritis. Nothing about the transgenic animals that we have generated, or based on the examiner's contentions in the Office Action, contradicts this conclusion.

9. Having thus discussed the components for creating a transgenic animal that demonstrates a phenotypic change characteristic of osteoarthritis during the interview, we next discussed that actually generating transgenic animals having these features is routine as of the time we made our invention. Any ordinary molecular biologist would have no trouble creating another mammal. particularly a rat, engineered to express an extracellular matrix degrading enzyme, particularly a type II collagen degrading enzyme, in a joint specific manner and under control of an inducible promoter. As further evidence of this fact, attached at Tab 4 is a copy of a paper by Bradley and Liu (Nature Genetics 1996, 14:121). This paper states (page 121, emphasis added):

> For almost 15 years the methods for making transgenic mammals have remained virtually unchanged, consisting of the injection of naked DNA into the pronucleus of a fertilized egg. The technique is so reliable that the technical shortcominngs can readily be circumvented by producing an excess of experimental material so that animals with the desired experimental coutcome can be selected from a collection of founder mice.

In short, contrary to the examiner's assertions, as of 1996 creation of transgenic mammals required no more than ordinary technical efforts – indeed, technical efforts with shortcomings

that are readily overcome. Bradley and Liu further discuss how expressing a transgene in embryonic stem (ES) cells provides an efficient and alternative genetic vehicle for transgene expression, especially for more complex transgenic constructs such as gene knockouts, creating chimeras with a dominant lethal phenotype, or for creating conditional mutants (page 122). Based on statements like these, it is clear to me that limiting our invention to transgenic mice based on the state of the art dramatically underestimates the actual state of the art. At the time we made our invention, making a transgenic animal was routine.

10. Given all of these features – regulatable and tissue-specific gene expression in transgenic animals, a variety of extracellular matrix degrading enzymes, particularly type II collagenase degrading enzymes, from which to choose, and acknowledgment of creation of transgenic animals as routine as early as 1996 (and, among researchers in the field, a whole lot earlier) – the only uncertainty remaining was to establish that this combination of features would cause phenotypic changes of osteoarthritis in a transgenic animal. These features are set forth on the slide attached at Tab 5, which was presented at the interview. Furthermore, as discussed at the interview, transgenic animals that have these features develop phenotypes of osteoarthritis, including changes in articular cartilage surface, cloning and disorganization of chondrocytes, osteophyte formation, synovial hyperplasia, and thickened subcondral bone (Tab 6). These features were found in two different transgenic lines that expressed MMP13* (constitutively active MMP13), Line 6 and Line 99, as demonstrated by Safranin O and H&E staining (Tab 6).[1]

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[1] Tet-regulated synthetic MMP13 genes and expression constructs were prepared and tested as described in Examples 1-5 (pages 33-44 of the Specification; my first Rule 132

11. The unique combination of technologies described in the Specification (see, *e.g.*, page 16, lines 7-13), *i.e.*, regulatable gene expression system and chondrocyte specific expression of a constitutively active extracellular matrix degrading enzyme, particularly a type II collagen degrading enzyme, has enabled development of a transgenic model resulting in lesion formation and other osteoarthritis pathologies (Tab 8). Lines 6 and 99, which expressed significant amounts of hMMP13*, showed osteoarthritis pathologies including lesion formation, cartilage degradation, and an inflamed synovium after induction of transgene expression (Tab 8). The crude models of joint degradation employed previously to establish animal models of osteoarthritis, including inbred mouse and guinea pig strains and ligament transection models (Tab 9, which was presented at the interview) further establish that this system, with well defined parameters of regulated expression of an extracellular matrix degrading enzyme in joint tissue, is enabled. In other words, if surgical or biochemical transection worked, there is a more than reasonable expectation after establishing the experimental data we have reported in mice that any transgenic mammal of the invention will work even better. Nothing about the transgenic animals that we have generated, or based on the examiner's contentions in the Office Action, contradicts this conclusion.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

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Declaration at paragraphs 5 and 8-11).

United States code and that such willful false statements may jeopardize the validity of the application or any patent issued thereupon.

LISA A. NEUHOLD, Ph.D.

Dated: Andover, Massachusetts
5/25/00, 2000

CERTIFICATE OF MAILING

I hereby certify that this paper and every paper referred to therein as being enclosed is being deposited with the U.S. Postal Service as first class mail, postage prepaid, in an envelope addressed to: Commissioner of Patents & Trademarks, Washington, DC 20231,

on April 6, 1999 (Date of Deposit)

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DECLARATION OF LISA A. NEUHOLD, Ph.D.,
UNDER C.F.R. § 1.132

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

LISA A. NEUHOLD, Ph.D., declares and states that:

1. I am a co-inventor of the above-identified patent application.

2. I presently hold a position as Senior Research Scientist I, Molecular Genetics, for Wyeth-Ayerst Research, Princeton, New Jersey, which is a division of American

Home Products Corporation, assignee of this application and the invention.

3. My qualifications as a scientist are set forth on the copy of my *curriculum vitae* which is attached as Exhibit A.

4. I have read and am familiar with the Office Action dated November 6, 1998. I understand that claims 1-17 and 22-27 have been rejected for lack of enablement. It is my understanding that the Examiner believes that the specification does not teach how to make transgenics in any other species other than mice and that the teachings of this application fail to provide a correlation between the effects of mutations in MMP, specifically MMP13, and cartilage-related diseases. The following data, which have been obtained in accordance with the teachings of this application, further demonstrate that the transgenic animal model of the invention is an effective animal model that mimics human osteoarthritis and related pathological syndromes and that the promoters utilized are chondrocyte specific and functional in a number of different mammalian cells.

5. Tet-regulated synthetic MMP13 genes and expression constructs were prepared and tested as described in Examples 1 and 2 (pages 33-38 of the Specification).

6. In addition to verifying the MMP13* activity, prior to microinjection, both transgene constructs in Examples 1 and 2 of the specification were tested in primary bovine chondrocytes, primary chick chondrocytes, mouse embryonic fibroblasts and HeLa cells. The constructs were not expressed in either the mouse fibroblast or HeLa cells and showed strong expression in both the bovine and chick chondrocytes (data not shown). The results demonstrate the ability of this rat collagen II promoter to induce expression of a second construct containing

either the Tet07-luciferase or the Tet07-MMP13* in a broad range of chondrocytes. The rat promoter is expected to be active in mammalian rat chondrocytes, is shown to be active in both mammalian bovine chondrocytes and avian chick chondrocytes. In addition, the data described in the specification and below demonstrates its strong activity in mammalian mouse chondrocytes. Thus, the constructs of the invention are specifically active in both mammalian and avian chondrocytes and are thus shown to be useful in the generation of the non-human mammalian transgenic animals of the invention.

7. Exhibit B, which corresponds to Figure 3 in the application, dramatically demonstrates tissue-restricted expression of a transgene under control of a regulatory system for use in the invention. Exhibit B-A is a diagram of the construct, in which the rat type II collagen promoter drives expression of *LacZ*, which is followed by a β-globin splice and a polyadenylation signal. Exhibit B-B is a photographic illustration corresponding to Figure 3B in the application of whole mount staining for β-galactosidase activity of embryonic day 16 transgenic mouse embryos expressing the transgene (see Figure 3A in the application). Blue staining is evident in the joints throughout the body of the transgenic animal, while no staining is observed in the non-transgenic, wild-type littermate. Specifically, joints including the ankles, knees, hips, phalanges, wrists, elbows, shoulders, and vertebrae. In addition to the cartilage of the joints, cartilage that has not ossified to bone at this stage of development, *i.e.*, some of the facial, skull, and rib bones also stained blue. These data confirm the expression abilities of the type II collagen promoter, and are useful in determining those tissues (joints) that will be expressing our MMP13* transgene. Exhibit B-C shows an enlargement of the elbow and paw

(this figure corresponds to Figure 3C in the application). These color figures are provided to clearly demonstrate that transgene expression in this regulated system is limited to the joint, and accordingly is highly relevant to osteoarthritis. Color images demonstrate these data effectively and dramatically.

8. The constructs were co-microinjected into fertilized mouse embryos (see Example 3, pages 38-39 of the Specification). Out of 112 newborn mice, 7 transgenic founders harboring both transgenes were identified, however, only four of these transgenic lines were capable of breeding. The transgenes were identified by PCR and verified by Southern blot analysis using a transgene-specific probe (data not shown). The copy number for each of the 4 transgenics was further assessed using Taqman quantitative PCR (data not shown). Briefly, transgene copy number ranged from 1-32 and 1-20 for the tet activator and MMP13*, respectively. Specifically, line 6 contained ~8 copies of the tet activator and ~3 copies of the MMP13* transgene. The remainder of the data in this Declaration focuses on the expression analysis of line 6.

9. Expression of the TA and MMP13* transgenes were initially evaluated in the hind-knee joints of four-month old mice by PCR (see Example 3, pages 38-39 of the Specification). Amplification of the c-fos endogenous cDNA was used as a control to verify the efficacy of each reaction. Exhibit C-A shows amplification of an 890 bp fragment resulting from a TA-specific primer set. Reverse Transcriptase-PCR (RT-PCR) showed the TA transgene to be expressed in transgenic mice both on and off Dox, but was not expressed in the non-transgenic controls (lanes 4-5). The method used for RT-PCR is described in the specification at page 42,

lines 2-6 in Example 5. Constitutive expression of the TA is expected since it is driven by a constitutively active collagen type II promoter. Moreover, expression of the TA is limited to the joints and was not observed by RT-PCR in other tissues including brain, heart, liver, kidney, spleen, or skeletal muscle (data not shown).

10. Exhibit C-B shows amplification of a 645 bp fragment resulting from an MMP13* specific primer set. Note, the MMP13* primer set is specific for human MMP13 and does not react with it's endogenous mouse homologue, collagenase-1. RT-PCR showed that MMP13* was not expressed in the non-transgenic controls (lanes 4-5). Lanes 6-7 show that there is expression of the MMP13* transgene in mice maintained on Dox. Removal of Dox from the drinking water induces a significant amount of expression (lanes 8-9). We have estimated the expressed amount in this transgenic mouse line (line 6) to correspond to a 3-4 fold induction. This induction was estimated using RT-PCR and titrating the amount of MMP13* cDNA. Exhibit D shows that amplification of 2.0 ul of cDNA made from a transgenic on Dox is required to obtain the same level of signal from 0.5 ul of cDNA made from a trangenic off Dox. Furthermore, following gel electrophoresis, PCR fragments were transferred to a nylon membrane and hybridized to a TA or MMP13* specific probe to verify the identity of the PCR product (data not shown).

11. To access any changes in the articular cartilage due to transgene expression, mice from line 6 were maintained or removed from Dox for 114 days, and their joints were sectioned and stained with hematoxylin and eosin (H&E) (see page 19, lines 4-6 of the Specification). When compared with an age matched littermate control, the transgenic removed

from Dox developed a pathology reminiscent of osteoarthritis (Exhibit E-B). The control animal showed no lesions or other osteoarthritis pathologies (Exhibit E-A), whereas the transgenic animal shows the formation of lesions in its articular cartilage (Exhibit E-B). More specifically, the H&E sections show considerable loss of cartilage, focal erosions, erosions that extend into the bone, and an inflamed synovium. Within the synovium there is evidence of fibroid necrosis, metaplasia, and synovial cell hyperplasia. In addition to these symptoms of osteoarthritis, some changes observed are more characteristic of rheumatoid arthritis. These changes include angiogenesis, as seen by an infiltration of red blood cells, monocytes, and macrophages. Exhibit E-C and E-D show the synovium at a higher magnification.

12. Tetracycline and their analogues are known inhibitors of MMP activity. As a result, we compared the serum levels of Dox when 1 mg/ml was added to the drinking water and the *in vitro* IC_{50}. In a MCA fluorescent assay the IC_{50} equals 59.1 μM, whereas the serum levels measured 2.64 μM using a zone of inhibition assay. These data show that the amount of Dox in the serum is 22.4 fold below the level at which 50% of MMP activity could be inhibited. Thus, it is unlikely that there is a significant inhibition due to the Dox.

13. The unique combination of technologies described in the Specification (see, *e.g.*, page 16, lines 7-13), *i.e.*, tetracycline regulatable gene expression system and chondrocyte specific expression of a constitutively active MMP protein, has enabled development of a transgenic model resulting in lesion formation and other osteoarthritis pathologies. Line 6, which expressed significant amounts of hMMP13*, showed osteoarthritis pathologies including lesion formation, cartilage degradation, and an inflamed synovium after

five months off Dox.

14. As observed in the line 6 transgenic, the joint destruction/erosion, lesions, fibroid necrosis, metaplasia, synovial cell hyperplasia and an inflamed synovium (in the absence of T-cells) are among pathologies observed in patients with osteoarthritis. However, not all of the pathologies observed in the transgenics are reminiscent of osteoarthritis. For example, angiogenesis and infiltration of monocytes and macrophages are pathologies observed during the inflammation process associated with rheumatoid arthritis. Note, the absence of neutrophils in the synovial fluid. Migration of neutrophils to the site of inflammation is a hallmark pathology of rheumatoid arthritis.

15. These data provide direct evidence that MMP13 is a critical player in the development of osteoarthritis. Moreover, the transgenics of this invention clearly provide an animal model to test the efficacy of therapeutics. Compounds that modulate the activity of MMP13 or inhibit progression of osteoarthritis can be monitored by determining lesion formation and other osteoarthritis pathologies at various times during the progression of the disease. Finally, the fact that the rat collagen II promoter drives expression in chick chondrocytes, bovine chondrocytes and transgenic mouse joints, combined with the fact that there is no expression from this promoter in either mouse embryonic fibroblasts or HeLa cells, demonstrates that the promoters of the invention can be used for expression in a tissue specific manner in a number of different mammalian chondrocytes (and even in avian chondrocytes) in the generation of the transgenic non-human mammals of the invention.

16. I further declare that all statements made herein of my own knowledge are

true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States code and that such willful false statements may jeopardize the validity of the application or any patent issued thereupon.

Lisa A Neuhold

LISA A. NEUHOLD, Ph.D.

Dated: Princeton, New Jersey
4/6, 1999

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ELSEVIER

TRANSGENIC LIVESTOCK: PROGRESS AND PROSPECTS FOR THE FUTURE

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ABSTRACT

The notion of directly introducing new genes or otherwise directly manipulating the genotype of an animal is conceptually straightforward and appealing because of the speed and precision with which phenotypic changes could be made. Thus, it is of little wonder that the imagination of many an animal scientist has been captivated by the success others have achieved by introducing foreign genes into mice. The private sector has embraced transgenic livestock technology resulting in the formation of two new industries. However, before transgenic farm animals become a common component of the livestock production industry, a number of formidable hurdles must be overcome. In this brief communication, the technical challenges are enumerated and possible solutions are discussed.

Key words: transgenic livestock, gene transfer, microinjection

INTRODUCTION

The definition of transgenic animals is evolving. For the purpose of this paper a transgenic animal is one containing recombinant DNA molecules in its genome that were introduced by intentional human intervention. In this review I will focus on animals in which transgenes were introduced into preimplantation embryos by pronuclear microinjection, with the intended consequence of producing germline transgenics as opposed to somatic cell transgenics. Though there are other means of introducing genes into preimplantation embryos (20,29), pronuclear microinjection, basically as originally described by Jon Gordon (25), and as modified for livestock in our laboratory (65), is still the predominant method employed.

Acknowledgments

Many of the concepts, conclusions and visions of the future included in this manuscript have evolved over the years from discussions at our Friday afternoon lab meeting. Vern Pursel and Caird Rexroad, Jr., who pioneered transgenic livestock technology, provided the leadership. In recent years Ken Bondioli, David Kerr, Paul Hyman and Uli Tillmann have provided valuable new insights and new approaches that have and will advance the field.

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WHY MAKE TRANSGENIC ANIMALS?

A Medline search reveals that over 6,000 scientific articles have been published in which transgenic animals (mostly mice) were used to answer basic research questions. By contrast 289 papers dealt with transgenic livestock, of which 24% were reviews. The limited publication record for transgenic livestock species reflects the high costs and technical difficulties associated with producing transgenic livestock more than lack of applicability of this technology to farm animals. A number of well defined goals have been enumerated in the numerous review articles written by animal scientists. Not surprisingly, many of the proposed applications closely parallel the long term objectives of animal agriculture.

In theory, transgenic technology provides a mechanism by which economically important traits can be attained more rapidly than by selective breeding without concern of propagating associated, possibly undesirable, genetic characteristics. If genetic precision and speed of improvement were the only advantages of transgenic technology, use of that methodology might be difficult to justify. That is because current cost of producing transgenic animals are high and understanding of the appropriate genetic manipulations required to influence economically important traits is limited. However, transgenic technology offers much more. Genes can be transferred across species boundaries and can be modified to function very differently than they do in their native form (gene products, tissue specificity, and timing of expression can be altered). The ability to redirect expression of genes to another organ has spawned the transgenic bioreactor industry. For the most part, transgenic bioreactors are farm animals designed to produce new proteins in their milk or other body fluids. It is envisioned that this approach will have application in both food production and the biomedical arena. Modifying the composition of milk through genetic engineering is the topic of Dr. Bremel's paper in these proceedings and will not be dealt with here.

TRANSGENIC LIVESTOCK PROJECTS

For the sake of brevity, only a very brief summary of the 37 gene constructs that have been tested in livestock will be reported here. The reader is referred to two excellent reviews that list those constructs and their consequences (16,53).

The Transgene.

The power of transgenic technology is derived from the introduction of genetic information with new functionality. The strategy for building a transgene (fusion gene) involves selecting a genetic regulatory element (often called promoters, but usually containing both an enhancer element and a promoter) that will determine the tissue in which the gene is to be expressed and the time and magnitude of expression. In some cases, the regulatory element can act as a switch, allowing the transgene to be turned on and off at will. The second part of the gene construct consists of DNA sequence encoding the desired protein (often referred to

as the structural component of the transgene). For example, in the first transgenic livestock experiment (28) we wanted to increase the levels of circulating growth hormone in a controlled manner. The gene construct used to accomplish this consisted of the regulatory element of a metallothionein (MT) gene fused to the coding sequence for growth hormone (GH). Metallothionein is an inducible liver enzyme, and its gene is usually quiescent (turned off) until a threshold level of circulating zinc or cadmium triggers transcription. Therefore, it was expected that the MT-GH fusion gene would be silent until the animals were fed zinc. In those experiments GH expression could be inducted but, in most cases, the transgene could not be turned off completely. New more complex inducible approaches are now being tested (23,26). These new systems rely on tetracycline or its analogs to activate or repress transgene expression. It is too early to know if these strategies, in their current form, will be more tightly regulated then the MT system. However, if they are not, the general paradigm on which the new systems are based will probably lead to improved inducible systems

Led Transgenic Projects.

The vast majority of original research reports have focused on growth enhancement. Growth hormone (GH) was the structural gene employed in 13 of those publications and the gene for growth hormone releasing factor in four. Other structural genes tested include IGF-1, cSKI and an estrogen receptor. The regulatory elements derived from MT genes, from various species, were most frequently used appearing in nine of the growth-related fusion genes. Long terminal repeats (LTR) from two retroviruses, MLV and RSV, and sequence from CMV, a DNA virus, served as regulatory components of transgenes, as have the promoters from albumin, prolactin, skeletal actin, transferrin and phosphoenolpyruvate carboxykinase (PEPCK) genes. All but two of 21 growth constructs were tested in pigs and the most striking phenotypes resulted from the use of MT-GH fusion genes (53).

Seven transgenes designed to enhance disease resistance and to produce immunologically-related molecules have been introduced into pigs and sheep (5,13,41,67). Though desirable expression patterns have been reported in several of the projects, none of the studies has progressed to the point of demonstrating a beneficial effect of transgene products.

Very recently it has been reported that transgenic sheep with enhanced wool production characteristics have been produced (9). The results are quite promising; if no unforeseen anomalies occur, transgenically produced wool maybe the first marketed livestock product.

Biomedical Transgenic Projects.

Other proposed transgenic farm animal applications are decidedly non-agricultural in nature. One of the first transgenic animal companies demonstrated the feasibility of producing new animal products by manufacturing human

hemoglobin in pigs, to serve as a principal component of a human blood substitute (59). Human antibodies have also been produced in transgenic mice (62). Another area where transgenic animals, especially pigs, will have a significant impact on society will be in the development of human genetic disease models. To date, genetic disease models have been generated in mice for atherosclerosis (6), sickle cell anemia (18), Alzheimer's disease (21), autoimmune diseases (44), lymphopoiesis (33), dermititis (55), and prostate cancer (61). These models for the most part require "knocking out" the function of a gene or replacing an existing gene with a mutant form. Many of these models will have to be replicated in farm animals to be useful. Unfortunately, the stem cell technology required to generate most of the disease models is still in development for livestock (51).

Finally, a new use not reported in the above mentioned reviews deserves note. The objective of this new endeavor is to genetically engineer animals, primarily pigs, so that their organs can be used as xenografts for humans. Preliminary studies to test the concept have been performed in mice (40,42) and transgenic pigs have now been produced (19,54). Though several strategies are being explored, the general approach has been to block activation of complement, which is normally part of the acute transplantation rejection response. These organs are intended for temporary use, until an appropriate human organ becomes available. However, as the technology develops, a driving force will be the design of transgenic organs for extended use or permanent transplantation.

CHARACTERISTICS OF TRANSGENIC ANIMALS

Transgenic livestock projects are costly, primarily because the process is inefficient. Production costs range from $25,000 for a single founder pig to over $500,000 for a single functional founder calf (64). The calculation for cattle was based on obtaining zygotes by superovulation of embryo donors, the normal practice for all mammalian species. However, the costs are reduced by as much as a third if oocytes derived from ovaries collected at slaughter are the starting material. The remainder of this review will be devoted to characterizing the transgenic animal model, to identify points in the process that reduce efficiency, and finally discussing possible approaches that have been proposed to overcome major hurdles to progress.

Transgene Integration.

Even though several hundred copies of a transgene are microinjected, any transgene that becomes incorporated into the genome generally does so at a single location. Exceptions are rare (58). Thus, transgenic founder animals are hemizygous for transgenes. It is also common for a transgene locus to contain multiple copies of the transgene arranged in a head-to-tail array. These two characteristics of transgene loci should provide clues to the mechanism by which transgenes integrate. So far, few researchers have formulated compelling hypotheses to explain the event (2,47) and the hypotheses that have been proposed remain untested

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to devise approaches t

Transgene inte
animals (cattle, sheep
and rats, Table 1).

Table 1. Examples of
several laboratories.

Species	Injected & transferred embryos (No.)	S
Mice	12,314	
Rabbits	1,907	
Rat	1,403	
Cattle[c]	1,018	
Pigs	19,397	
Sheep	5,424	

[a] Number of experiments. v
tested.
[b] The value for cattle inclu
[c] Eleven thousand two hu
eighteen developed to mor

Transgene Expression

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Without knowledge of the molecular mechanism it is going to be extremely difficult to devise approaches to make transgene integration more efficient.

Transgene integration efficiency is low and ranges from about 1% in farm animals (cattle, sheep and pigs) to about 3% in laboratory animals (mice, rabbits and rats, Table 1).

Table 1. Examples of embryo survival and transgene integration efficiencies from several laboratories.

Species	Injected & transferred embryos (No.)	Studies[a] (No.)	Transgenic animals produced: Offspring[b] (No.)	Per Offspring (%)	Per embryo injected & transferred (%)	Refs.
Mice	12,314	18	1847	17.3	2.6	(63)
Rabbits	1,907	1	218	12.8	1.5	(28)
Rat	1,403	5	353	17.6	4.4	(45)
Cattle[c]	1,018	7	193	3.6	0.7	(30)
Pigs	19,397	20	1920	9.2	0.9	(53)
Sheep	5,424	10	556	8.3	0.9	(53)

[a] Number of experiments, which in most cases was equivalent to number of different gene constructs tested.

[b] The value for cattle includes both fetuses and live born calves.

[c] Eleven thousand two hundred and six eggs were microinjected and cultured. One thousand and eighteen developed to morula or blastocysts and were transferred into recipient cows.

Transgene Expression

Even after the one in 33 to one in 150 injected and transferred eggs results in a transgenic animal the efficiency of the process is further diminished by failure of the transgene to be transcribed. Transgenes are expressed (transcribed) in only about half of transgenic lines, though some specific transgenes are expressed in a higher proportions (15 ?7). If a founder expresses its transgene, so do its transgenic offspring. It is not clear why some transgenes are expressed in all lines and others in only half the lines. Transgenes are sometimes activated in unintended tissues (ectopic expression), and timing of expression can be shifted relative to development. Our lack of understanding of essential genetic control elements makes it difficult to design ...nsgenes with predictable behavior. The abbarent expression patterns (no expression or wrong expression) seen in some linese of transgenic animals has been attributed to the so-called "position effect." If a transgene lands near highly active genes, the transgene's behavior maybe influenced by endogenous genes. Other transgenes may locate in transcriptionally inactive (heterochromatin) regions. The transgene may function normally or be completely silenced by the

heterochromatin. It is likely that both of these factors (position effect and unidentified control elements) contribute to lack of transgene expression in some lines and variable expression in other lines. Some of these problems will be obviated by use of "boundary" DNA sequences that block the influence of surrounding genes (34,43). Refining transgenic technology for farm animals will remain a challenging task in part because experimentation will often have to be conducted in the species of interest. That is because transgene expression and the physiological consequences of transgene products in livestock are not always accurately predicted in transgenic mouse studies (28,48).

Transgene transmission.

Because founder animals are usually single integrant hemizygous for the transgene, one would expect 50% of their offspring to inherit a copy of the transgene locus. This is true for about 70% of transgenic founder mice (49). The remaining founders either do not transmit transgenes to their offspring or transmit transgenes at a low frequency (52,53). It is commonly thought that the non-Mendelian inheritance is the result of transgene mocasicism in germ cells. This could be caused by late integration of transgenes during embryonic development (60). It has been proposed that non-Mendelian inheritance patterns can also be caused by diminished fertilizing ability of transgene bearing sperm (17). The latter explanation may be a special case, because the thymidine kinase gene used in that study was inadvertently expressed in testes.

POTENTIAL SOLUTIONS FOR IMPROVING EFFICIENCY

Testing Transgenes.

Because the "rules" for transgene design are still vague, it is important to have a reliable system for testing gene constructs. The most cost effective method of characterizing the performance of a transgene is cell culture transfection studies. Unfortunately, such studies have a low predictive value (50). The next most cost effective method for testing gene constructs is production of transgenic mice, which as mentioned above do not faithfully predict a transgene's performance in livestock species. Nevertheless, a reasonable amount of useful information about transgene function can be derived from transgenic mouse studies. Currently, the only approach that yields truly informative data is testing transgenes in the livestock species of interest. This is obviously an unsatisfactory, time consuming, expensive testing option. One alternative approach that we are exploring is based on the fact that transgenes will function after being "shot" into somatic tissue. We have been focusing our efforts on the mammary gland, but almost any target organ should be amenable to this approach. We have recently demonstrated that both RNA and protein can be detected following introduction of transgenes into sheep mammary tissue, *in situ* (22,37). Once we confirm that "gene-gunned" transgenes function as they do in transgenic animals, this approach should dramatically reduce the costs and time of evaluating gene constructs.

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Improving Integration Frequency

From Table 1 it is clear that integration rates are lower for livestock species than for laboratory animals. Eggs of livestock species are more difficult to microinject than eggs of laboratory animals. However, competent microinjectors can reliably inflate pronuclei with DNA-containing solutions. Furthermore, integration problem occurs after the transgene is deposited. But timing of microinjection may contribute to differences in integration efficiency. It is thought that transgene integration occurs during DNA replication (2), so it would be advantageous to microinject before or during early S-phase preceding the first mitotic division. For the most part that is when laboratory animal eggs are microinjected, but microinjections are apparently performed during late S-phase or later in livestock species (for a full discussion see (63)). Efforts to inject *in vitro* fertilized bovine zygotes early have failed because of difficulties in visualizing pronuclei (K. Bondioli, personal communication and unpublished data). Efforts to synchronize microinjection and S-phase in bovine zygotes have thus far not been fruitful (24).

One way to insure that the transgene is in place before the first mitotic S-phase is to introduce the transgene at fertilization. That could be achieved by sperm-mediated gene transfer (4,38). Notwithstanding the controversy this approach has generated (8), it clearly represents an intriguing method that shows some promise (57). Accumulating evidence suggests that sperm of several species can bind transgenes (11,32,39,68) and carry the genes into oocytes where in some cases the gene persists (4,12,31). However, it appears that in almost all cases, the transgene DNA becomes rearranged or otherwise mutated by the process (Corrado Spadafora, personal communication). Another potential sperm-based delivery approach has been foretold by a pioneering study conducted by Ralph Brinster (7). In that study, transplanted spermatogonial cells generated sperm capable of fertilizing oocytes and offspring were produced. If a means is found to culture, transfect and select spermatagonia with transgenes, Brinster's transplantation scheme could be used to produce transgenic animals. Others have proposed directly transfecting testes as a means of transforming sperm (56).

Retroviral-mediated gene transfer is also a potentially alternative approach for introducing transgenes into embryos with high efficiency (29,36). Though the technique solves the low integration frequency problem, it creates other inefficiencies by generating mosaic founders that may not transmit their transgene. Furthermore, retroviruses can carry only a limited amount of exogenous DNA and therefore the technique limits the size of transgenes. If cDNA based transgenes, which are relatively short, were efficiently expressed, the transgene size restriction would not be a significant problem. However, many cDNA based gene constructs are poorly expressed in transgenic animals (66).

Selection of transgenic embryos.

With no obvious or immediate solution for improving integration frequency, what else can be done to increase efficiency of producing transgenic livestock? One of the most widely discussed approaches is selection of transgenic embryos before they are transferred to recipients (1,14,35,46). If transgenic preimplantation embryos can be identified by analyzing embryo biopsies with the polymerase chain reaction (PCR), the number of recipients required could be greatly reduced. For example in Dr. Bondioli's study ((30). Table 1), 1,018 bovine embryos were transferred into over 1000 cows resulting in seven transgenic calves and fetuses. If embryo selection had been possible, fewer than 20 recipients would have been required. Unfortunately, mounting evidence suggests that this approach will not work. In two very similar studies (10,14) microinjected mouse embryos were cultured to the 8-cell stage, and blastomeres were isolated and analyzed for the transgene by PCR. In our study (10) none of the 8-cell embryos had transgenes in more than 4 blastomeres. We speculate that immediately upon microinjection, transgene copies join to form multi-copy circular arrays. One of these arrays may eventually become integrated, while the non-integrated arrays segregate as daughter blastomeres are formed. If integration occurs after the one-cell stage, some blastomeres may not contain an array, even though the embryo is transgenic. The converse is also possible (all blastomeres acquire arrays but none integrate). Analysis of embryo biopsies could therefore be misleading.

Another scheme for selecting transgenic embryos before transfer is based on expression of a selectable marker-containing transgene. The preliminary results from two recent studies (3,60) appear to be promising. In both studies, transgenes containing a neomycin resistance gene (neo) were microinjected into pronuclei of mice (60) or bovine (3) embryos. The embryos were then cultured in the presence of G418, a neomycin analog, in the hope of killing embryos that did not express the neo gene. Because this approach is based on gene expression and because transgenes can be expressed without being integrated, embryos containing unintegrated copies of the transgene could survive the selection process. However, since G418 interferes with protein synthesis, the blastomeres that expressed the neo gene would have a developmental advantage over those that did not. Therefore, the blastomeres expressing the neo gene might divide more rapidly and have a higher probability of participating in the formation of the inner cell mass (66). Further studies will have to be conducted to determine if this scheme has merit.

In The Future

The tools for gene transfer are in hand, albeit the process is inefficient. Over the next decade, bioreactor and xenograft industries will mature and useful new products will be marketed. The value of possible products will drive the technology as funding for basic research from conventional sources becomes increasingly limited. Researchers will need to develop a better understanding of how mammalian genes are controlled, and identify key genes in regulatory pathways of

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phenotypic characteristics that are to be altered to bring the fruits of this technology to animal agriculture. There is a serious need to transfer transgenic animal technology from a few practitioners to many more laboratories worldwide. Progress in the field will be limited as long as the capabilities to explore this potentially powerful tool is only in the hands of a few. To entice other scientists, the efficiency of producing transgenic farm animals will have to be improved. But the horizon looks bright. Many recently trained animal scientists are now equipped with the knowledge and technical skills needed to advance this technology.

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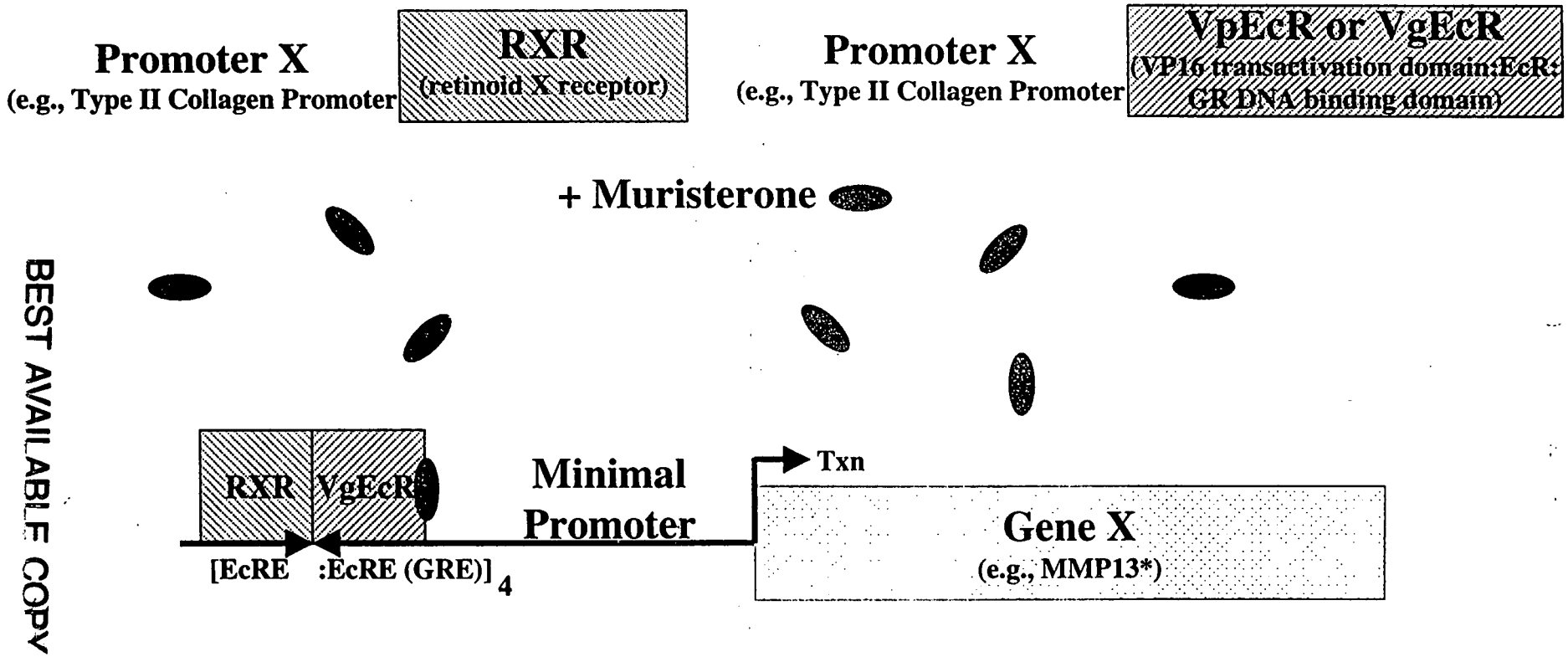
Ecdysone Inducible System

- Heterodimeric steroid-inducible system
- Wt system
  - EcR (ecdysone receptor): transactivating protein
  - USP (ultraspiracle protein): ligand binding protein

- Modified System

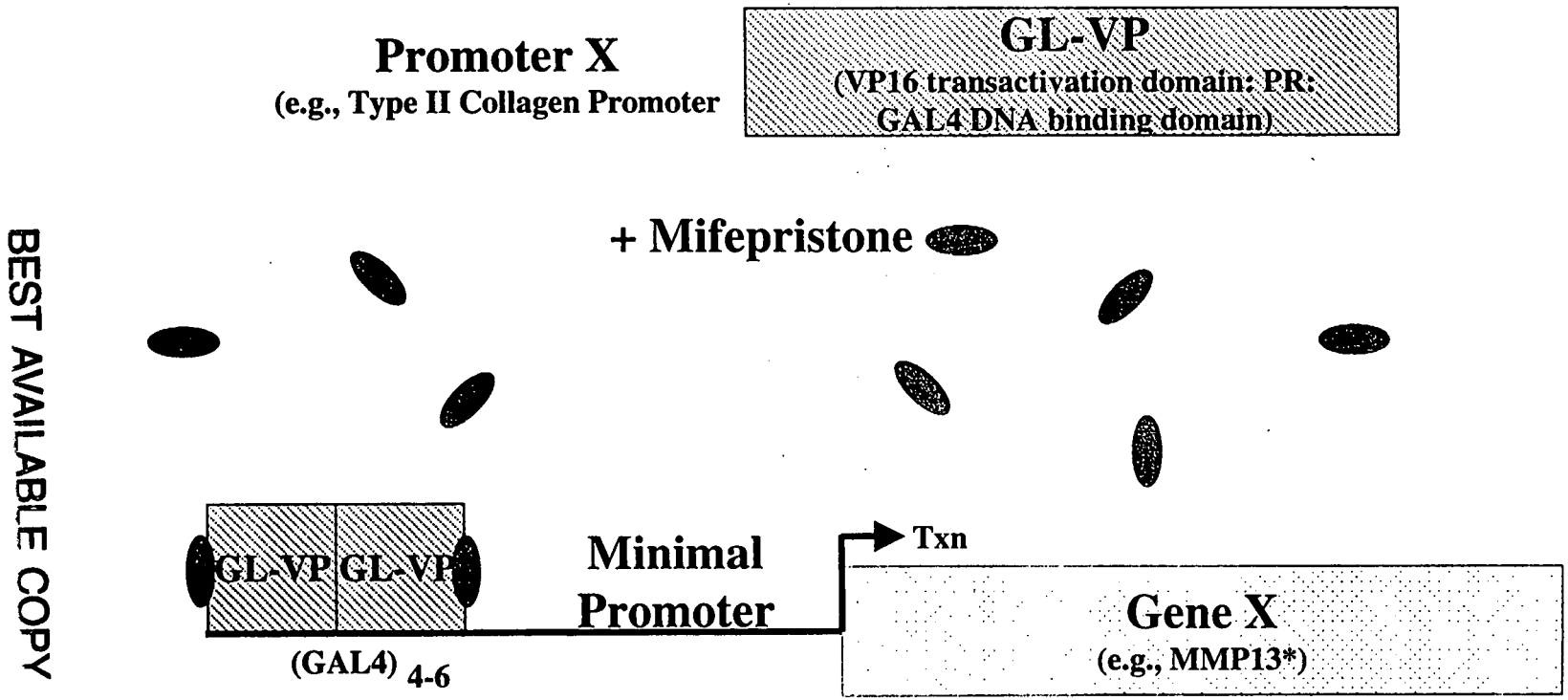


RU486 Inducible System

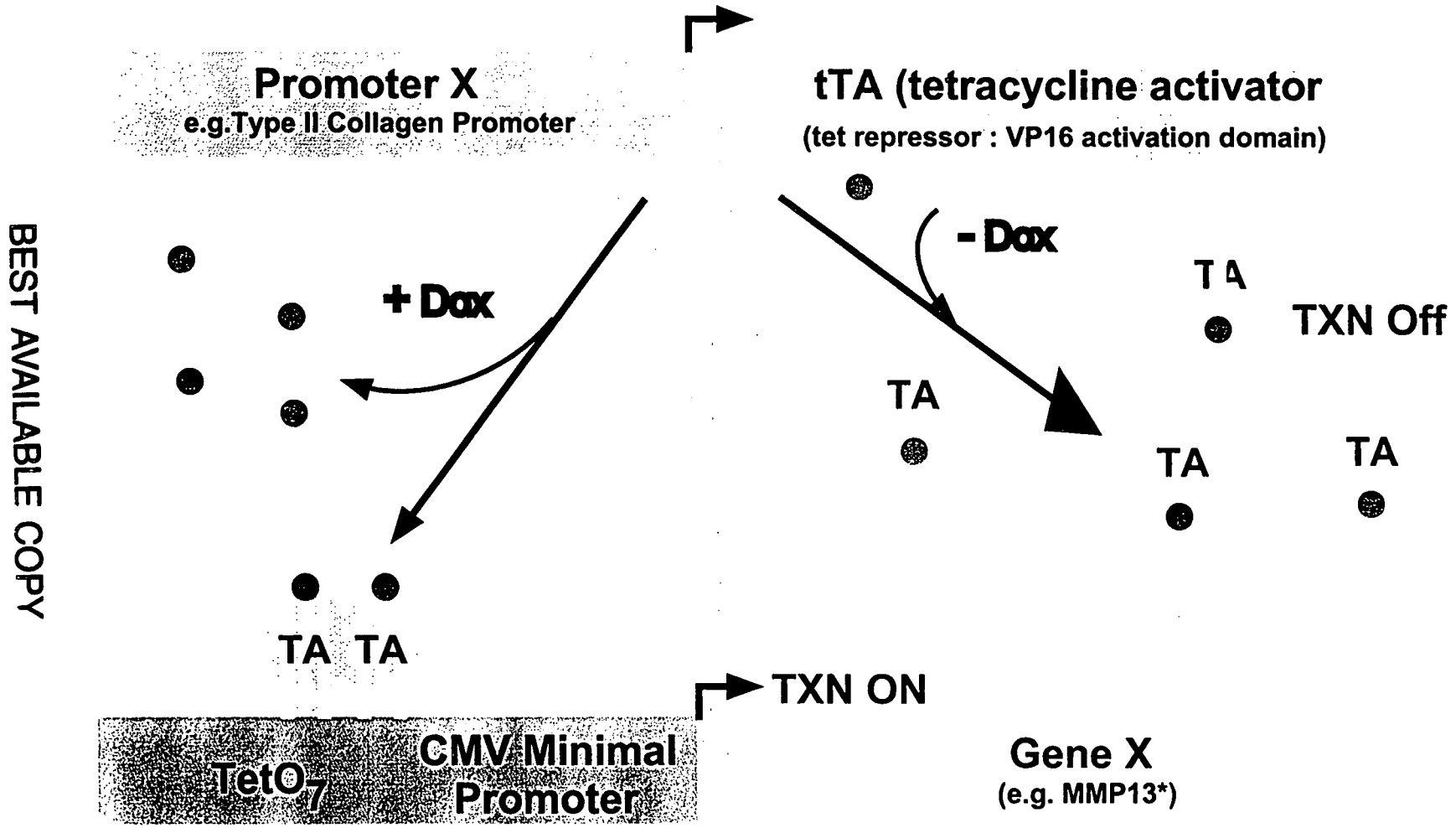
- Monomeric dimer steroid-inducible system
- Wt system

  Progesterone Receptor: induced by progesterone and mifepristone (RU486)

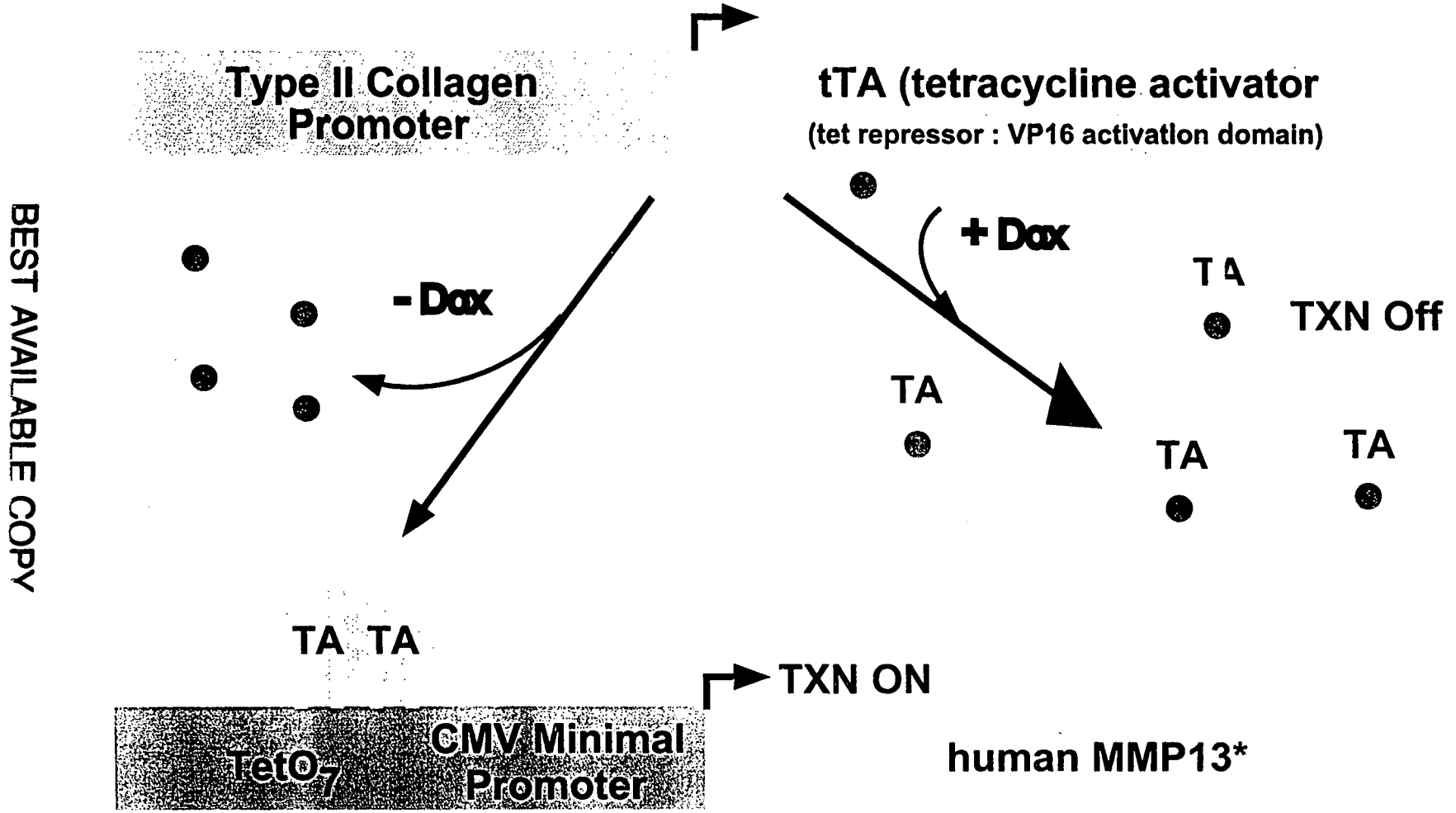
- Modified System



Schematic Diagram of the Reversed Tetracycline Regulated (Inducible) System



* Schematic Diagram of the Original Tetracycline Regulated (Repressible) System



During my literature search for alternative promoters that could have been used to drive MMP-13* instead of the type II collagen promoter I discovered the CD-RAP/MIA gene (1). Note, CD-RAP and MIA are the same gene.

The CD-RAP protein, also referred to as the cartilage-derived retinoic acid sensitive protein, is expressed throughout chondrogenesis and is co-expressed with type II collagen mRNA (2). Specifically, expression of CD-RAP was shown in 11.5 to 16.5 day old embryos via in situ hybridization to coincide with the type II splice form of type II procollagen mRNA that is found in articular cartilage. Therefore, the CD-RAP promoter would be a good substitute for the type II collagen promoter. Moreover, the promoter region of CD-RAP shares several potential regulatory domains with the type II collagen promoter.

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Transgenic Related Models Expressing MMPs

- Membrane bound MT1-MMP deficient mice were shown to develop several connective tissue defects including arthritis. However, the arthritis observed resembles the CIA model and human RA (Holmbeck et al., 1999)
- Stromelysin (MMP-3) deficient mice showed no change in their susceptibility to collagen-induced arthritis (Mudgett et al., 1998)
- Expression of a gene targeted, collagenase-resistant type II collagen results in connective tissue defects but not OA (Lui et al. , 1995)
- Expression of constitutively active MMP-1 and stromelysin in mammary tissue (Witty et al., 1995)
- Expression of human interstitial collagenase (MMP-1) in the lung (D'Armiento et al., 1992)
- Expression of IL-1 signal sequence fused to hMMP-1 in the articular cartilage of mice (unpublished, W. Horton)

nal chromoson ...n the XX germline, a BD-encoded i... ...ictor (BD imprintor) activity on th ...ternally inherited chromosome is required to 'prime' the paternal chromosome via an interaction at exon 1. This makes the chromosome accessible to a *trans*-acting factor specific to the female germline that results in the chromosome acquiring a maternal epigenotype. In their model, the maternally inherited chromosome retains the maternal epigenotype. In AS lineages, the mutant BD-imprintor on the paternal allele would not operate; thus, the *trans*-acting factor would not have access to the 'unprimed' paternal chromosome, so a maternal epigenotype switch could not be acquired. It is, however, more difficult to explain maternal to paternal switching in the XY germline. The authors suggest that in the absence of the *trans*-acting factor, the paternal epigenotype is retained on the paternal chromosome and that, even in the absence of BD activity, the maternal chromosome acquires the paternal epigenotype by default. This has implications for exon 1 function in the XY germline in PWS lineages where exon 1 mutation on the maternal chromosome fails to allow the acquisition of the paternal epigenotype. This suggests that exon 1 is required for this default activity. Another possibility, however, is that in PWS, failure of imprint erasure may be occurring and that exon 1 may be involved in the erasure process, and this makes a single step switch less likely than one that involves both an imprint erasure and a germline-specific resetting.

In the mouse, some evidence ...ts for a mechanism involving bot... ...sure and re-establishment of ... imprints in the germline[17]: *Snrpn* is biallelically expressed in germ cells of both male and female mice[18]. If exon 1 is required for first erasing the imprint, one could invoke a two-step model (see Fig. 2 for modified model of that proposed by Dittrich *et al.*) in which erasure occurred, then biallelic BD-imprintor activity, necessary for acquisition of the maternal epigenotype in the XX germline, would take place. Absence of BD activity would then be required for the paternal epigenotype in XY germlines. In PWS, exon 1 mutations could not erase imprinting, regardless of whether they are in a male or female germline — however, for maternally inherited mutations in a XX germline, this doesn't matter as they will always have the appropriate maternal epigenotype. In the XY germline this will lead to the inability to switch. While in this case a maternal-specific *trans*-acting factor is not required, a means to regulate BD activity in an XX germline specific manner must be assumed. The paternally inherited chromosomes can later activate BD transcripts in the tissues of the fetus, whereas they remain silent on the maternally inherited chromosome.

Conclusions

Taken together, the studies of Hatada *et al.* and Dittrich *et al.* focus our attention on the clustered organisation of imprinted genes into large domains and the significance this organisation may have on both functional and mechanistic aspects of genomic imprinting. BWS, AS and PWS now show some common features, such as mutations in the key germline events, mutations in regional control, and now for BWS, mutations in an individual imprinted gene. Mutations in an individual gene are also supected for AS because IC mutations and UPD are rare[15]. As a result, the whole process of genomic imprinting can be dissected at many different levels and shows promise for understanding how (and maybe even why) these mechanistic and functional controls affect phenotype. Much is being learned from human genetic analysis, but it is clear that further experiments in the mouse must be carried out to test the efficacy of the models these studies continue to generate.

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Target practice in transgenics

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For almost 15 years the methods for making transgenic mammals have remained virtually unchanged, consisting of the injection of naked DNA into the pronucleus of a fertilized egg. The technique is so reliable that the technical shortcomings can readily be circumvented by producing an excess of experimental material so that animals with the desired experimental outcome can be selected from a collection of founder mice[1]. Several recent reports, however, utilize methods for expressing a transgene in embryonic stem (ES) cells instead of direct zygote injection as an alternative — and sometimes the only — means for analysing gene expression in mice.

Injection of DNA into a mouse zygote pronucleus has two predictable outcomes, namely that the DNA will concatamerize (head-to-tail) and that it will integrate into the genome[2]. Herein lie several of the variables with the method. First, the integration site of the DNA is unknown — and the insertion site can strongly influence the expression of the integrated DNA. Second, the number of integrated copies of the injected DNA will vary widely, and this can affect transgene expression. Third, for a transgene to recapitulate faithfully the expression of the endogenous gene, it is essential that all of the appropriate positive and negative elements of the gene are present. Since the position of these elements vary, trial/error is required to identify them so that they may be included as a compo-

nent of the transgene; elements that are located a great distance from or are within a large gene are particularly difficult to identify[3].

The increasing acceptance of ES cells as an efficient and alternative genetic vehicle for transgene expression has begun to facilitate experiments not possible by direct zygote injection and to remove some of the uncertainty regarding the expression of transgenes. Although ES cells have principally been used to establish loss-of-function mutations (gene knockouts), the use of ES cells for transgene expression pre-dates gene targeting in ES cells[4]. These decade-old experiments established one of the important advantages of ES cells for transgene expression — namely that if expression causes a dominant lethal phenotype (making it impossible to obtain such mice by zygote injection), useful expression data could still be obtained because the transgenic cells are usually rescued in a chimaera. Moreover, germ-line transmission of the transgene is not required because a cohort of animals for experimental analysis can be rapidly generated from the same ES cell clone by repeated blastocyst microinjection.

In a recent paper by Corral *et al.*[5], the 'classical' experimental approach of generating and analysing chimaeras was used; however, in this instance, the ES cells were modified to express a fusion gene. Fusion genes were first used in ES cells as a means to select for targeted clones (see Fig. 1*a*) in the days when homologous recombination was believed to occur inefficiently[6]. In the Corral *et al.* study the precision of homologous recombination was used to generate an in-frame fusion by targeting the 3' half of human *AF9* to the *Mll* locus. In this way the *AF9* gene was brought under the transcriptional control of the *Mll* locus and this mirrored the *Mll–AF9* fusions found in human leukaemia. Descendants of these ES cells contributed widely to the chimaeras, and these animals developed leukemia, confirming the functional significance of the *Mll–AF9* fusion in human disease. Another type of fusion gene was generated between β-galactosidase and *Mll*, facilitating the analysis of both the expression of the *Mll* locus and the subcellular location of Mll fusions[6]. The targeting of β-galactosidase coding sequences is an excellent way to define the expression patterns of a gene. The widest use of this method has been in gene trap experiments[7], where β-galactosidase expression has been used as a means to identify genes with 'interesting' patterns of expression[8]. While β-galactosidase is being widely used as a reporter gene for expression analysis, green fluorescence protein (GFP) provides a potentially attractive alternative because expression may be evaluated in living tissue. Additionally, site-specific recombinases (such as FLP and Cre) represent another type of gene product that will almost certainly be targeted to many different loci for the purposes of generating tissue-specific knockouts[9].

Targeted transgenes are beginning to be used for purposes other than expression analysis. One elegant use of this system has been to determine whether different members of gene families are capable of functional compensation (Fig. 1*a*). During evolution gene family members diverge both with respect to nucleotide composition and to temporal/spatial patterns of gene expression. At some point the two genes will be sufficiently divergent that one can no longer compensate for the function of the other — but is this the result of sequence divergence or differences in expression pattern or both? This type of question has recently been resolved for two pairs of genes (*engrailed* 1/2 and *myogenin/myf5*) by targeting one gene to the locus of its homologue and observing rescue of a knockout phenotype[10,11].

There are some situations where the *cis* elements required for tissue specific expression have been relatively well characterized. Under such a circumstance targeting a transgene to another locus can be considered an unnecessarily elaborate experimental protocol compared with pronucleur injection. However, with injection, the copy number and integration site remain as significant variables. This type of unpredictability can be avoided by using ES cells as the genetic vehicle. It is possible to prescreen transfectants for the desired copy number or to deliver the transgene to a defined locus by homologous recombination (Fig. 1*b*). In a recent paper by Bronson *et al.*[12], the use of *Hprt* as a locus for transgene integration was described. The *Hprt* gene was actually the first locus to be mutated in ES cells[13] and

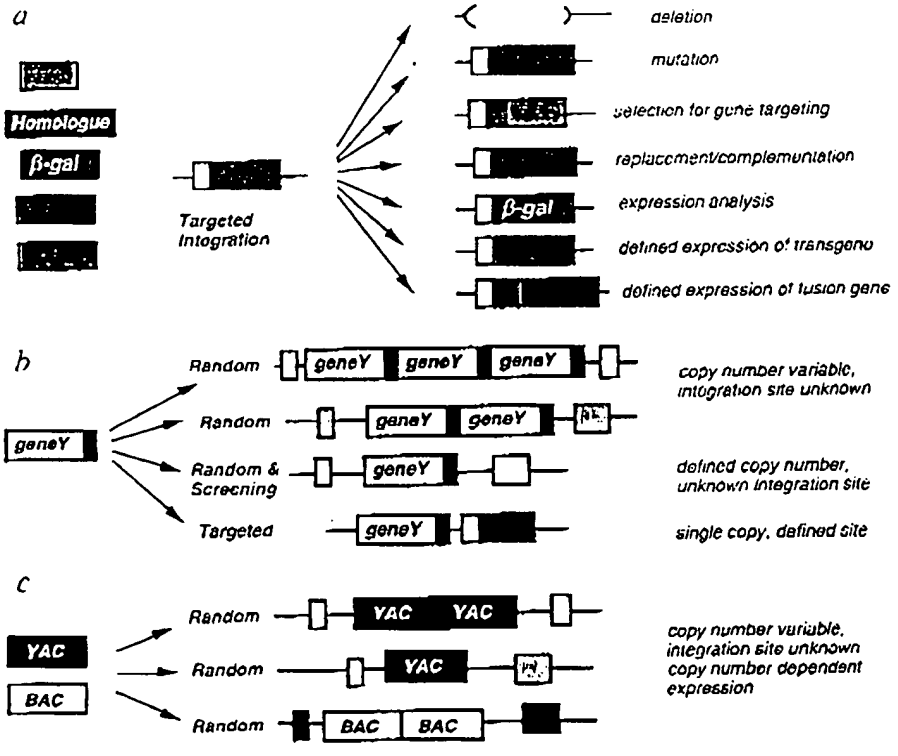

Fig. 1 a, Utilizing position effects to study gene expression, whereby targeted transgenes (including fusion genes and gene family members) mirror expression of the endogenous gene. *b*, Controlling transgene position effects using selection of integration loci and copy number in ES cells. c, Controlling transgene position effects with BACs and YACs.

established as a mouse strain via ES cell technology, and, like many subsequent observations on knockout mice, the phenotype was not predicted: the mice (unlike humans) were not dramatically affected by the absence of *Hprt* function. Given that both loss- and recovery-of-function mutations in *Hprt* can be directly selected in XY ES cells in culture, selection for gene targeted events is readily achieved. In the Bronson *et al.* study, the ES cell line carried a mutant *Hprt* allele that is restored by gene targeting. The *Hprt* locus is not the only choice for this type of experimental strategy, and many transgenes must be examined in detail before it can be concluded that the regulatory elements in the *Hprt* locus do not influence transgene expression. Given that *LoxP* sites have been targeted to many loci in the mouse genome, these alleles are likely to be evaluated as possible preferred integration loci for transgenes, using Cre to stimulate site specific integration.

One disadvantage of targeted delivery of transgenes to specific loci is size limitation. Thus the transgenes generally need to be exceptionally well characterized with respect to *cis* elements required for appropriate temporal, tissue and spatial expression. As an alternative, non-targeted delivery of large DNA fragments can be used since these are usually relatively inert to position effects. The methodology for constructing transgenics with YACs and P1 BACs is now firmly established (Fig. 1*c*). Because of the large physical size of these vectors, they usually contain most if not all of the regulatory elements of the gene of interest, and consequently, expression is usually integration site independent. From a technical perspective transgene delivery is simple and may be accomplished by direct pronucleur injection of zygotes or ES cell lipofection[14]. ES cells offer the advantage that a few selected transfectants may be analysed from a larger set of independent integration events. From a genetic perspective, large transgenes facilitate study of the effect of gene dosage, given that genes expressed from large transgenes are frequently copy number dependent as well as integration site independent[15].

The richness of the repertoire of the genetic modifications that can now be established in the mouse germ line via ES cells is impressive and will only increase. Although most mutations have been generated on the 129 genetic background, the availability of ES cells from other strains such as CBA[16] (see page 223) will facilitate the choice of the most suitable inbred background for phenotypic analysis. Moreover, generating the same genetic lesion in two different backgrounds will enable the identification of enhancer/modifier loci. In the future it is likely that gene targeting will be surpassed by an increase in the number of high-throughput procedures of gene disruption to define gene function. On the gene discovery side, chromosomal changes[17] are being engineered for the purposes of phenotype driven screens, and complementation of recessive mutations by YACs and BACs will replace large mouse crosses to finely map genes. The challenge with ES cells is no longer technical but resides where it should, in one's ability to ask the right question. ES technology is intoxicating, and we should all satisfy our thirst.

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Mitochondrial DNA gets the drift

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Mammalian mtDNA has provided a wealth of interesting molecular puzzles since its discovery almost 30 years ago. Among these are its distinctive modes of replication and expression, its use of a modified genetic code, the unorthodox features of its transcripts and the unpredicted existence of phage-like mitochondrial polymerases[1]. It has been known for some time that although mammalian mtDNA likely exists at high copy number in most cells ($\sim 10^3$–10^4 copies/cell), each individual in a given species is essentially homoplasmic for mtDNA (that is, virtually all of an individual's mitochondria have the same DNA sequence); any mtDNA sequence variation in normal individuals is localized primarily to the displacement-loop control region[2]. However, mtDNA sequence variations *between* individuals are clearly prevalent, and the presence of such polymorphisms has provided a basis for classifying human populations and their evolution, and has also provided a tool in forensic studies for identification of individuals.

A central question surrounding mammalian mtDNA segregation is how to explain the rapid selection of mitochondrial genotypes observed in pedigree analyses in different mammalian species; a change in mtDNA sequence in a pedigree generally can be seen to occur within a single generation in humans. The two favourite hypotheses that have been advanced to explain this phenomenon focus on mtDNA replication and on possible physical partitioning. If early in embryogenesis or in the female germline (mammalian mtDNA is maternally inherited) there was selective amplification of

Osteoarthritis (OA)

- Degenerative joint disease involving the destruction of articular cartilage
  - proteogylcan loss
  - a decrease in the number of chondrocytes
  - loosening/flaking of the cartilage surface
  - loss of cartilage

- Primary target is the degradation of type II collagen

- MMP (Matrix-Degrading Metalloproteinases) Family (~24 members)

  3 classes: collagenases
  stromelysin
  gelatinases

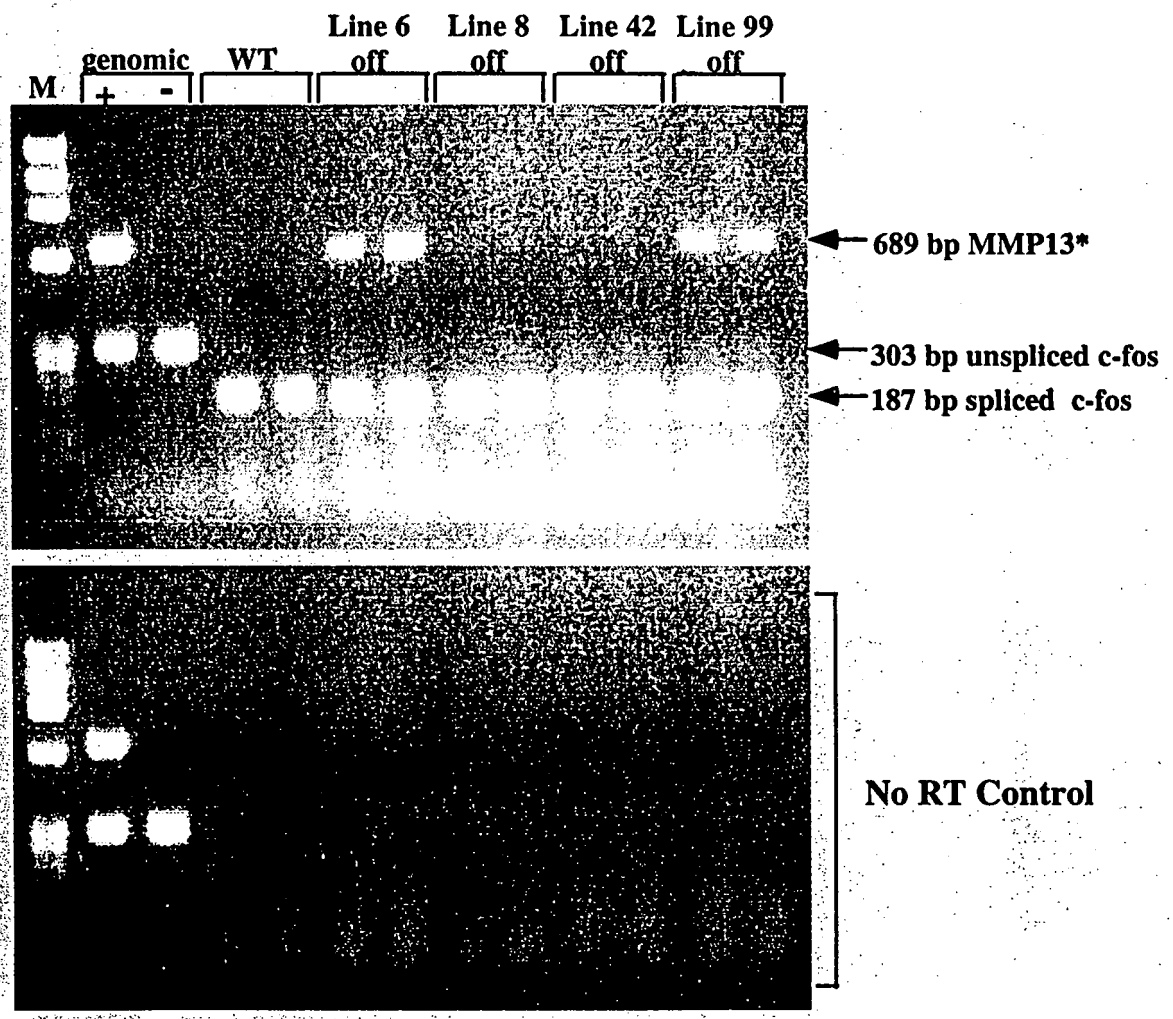
- MMP13 (collagenase-3)
  - Type II collagen is an excellent substrate for digestion by MMP13 and a weaker substrate for MMP-1
  - MMP-13 can be detected immunohistochemically in human arthritic tissue
  - Levels of MMP-13 are significantly higher that MMP-1 in human arthritic tissues

Phenotype of MMP13 Transgenic Mice

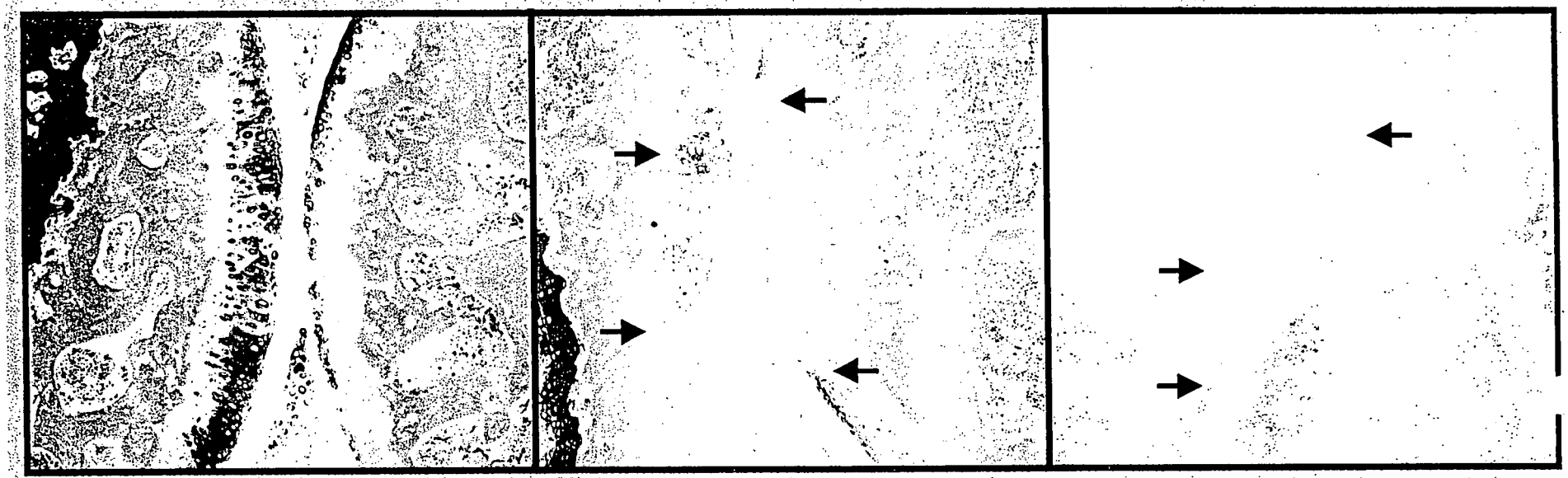
Osteoarthritis	MMP13* Transgenic Phenotype
articular cartilage surface:	
fibrillation/fissures	✓
ulceration/erosion	✓
disintegration/loss of chondrocytes	✓
lesions	✓
cloning/disorganization of chondrocytes	✓
osteophyte formation	✓
synovial hyperplasia	✓
thickend subchondral bone	✓

Rheumatoid Arthritis	
systemic disease	
degenerative joint changes	
synovium	
chronically & highly inflamed	
new vessel formation (angiogenesis)	
macrophage infiltration	
chemotaxis of neutrophils & T-cells	
thickening of synovial lining & proliferation	
PANNUS formation	
villous projections	
intra-articular bleeding	
ankylosis	
rheumatoid nodules	

Expression of MMP13* in Different Transgenic Lines



Safranin O Staining of the Articular Cartilage from the Hind Knee Joint

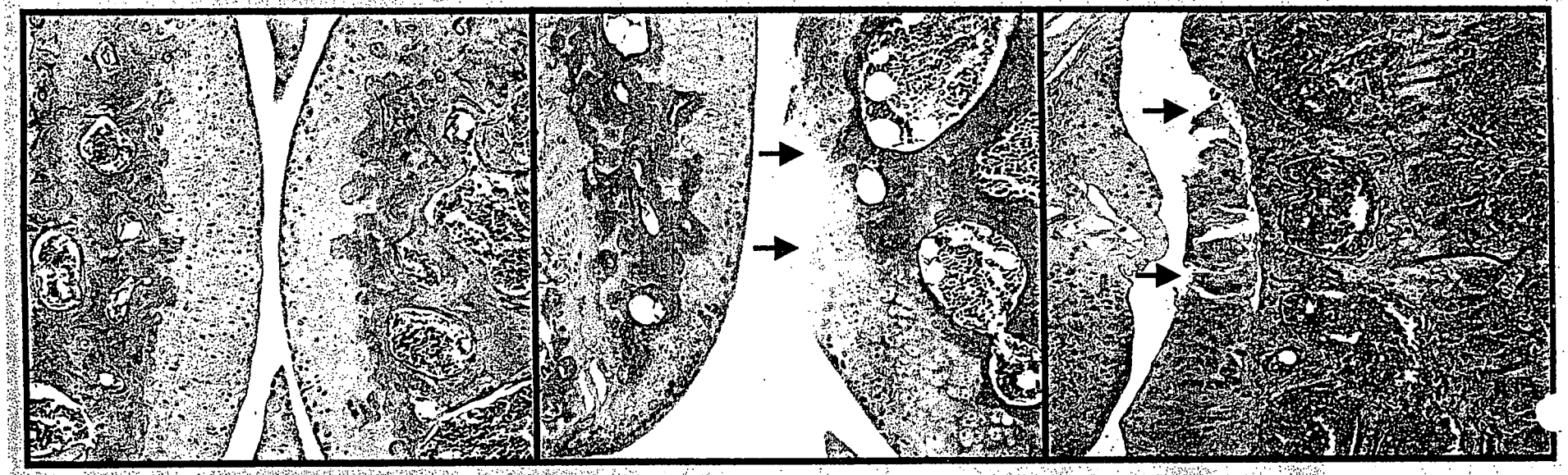


Wild Type
(5 mos)

Line 6-1
(5 mos)

Line 99-1
(5 mos)

H&E Staining of the Articular Cartilage from the Hind Knee Joint of an Adjacent Section



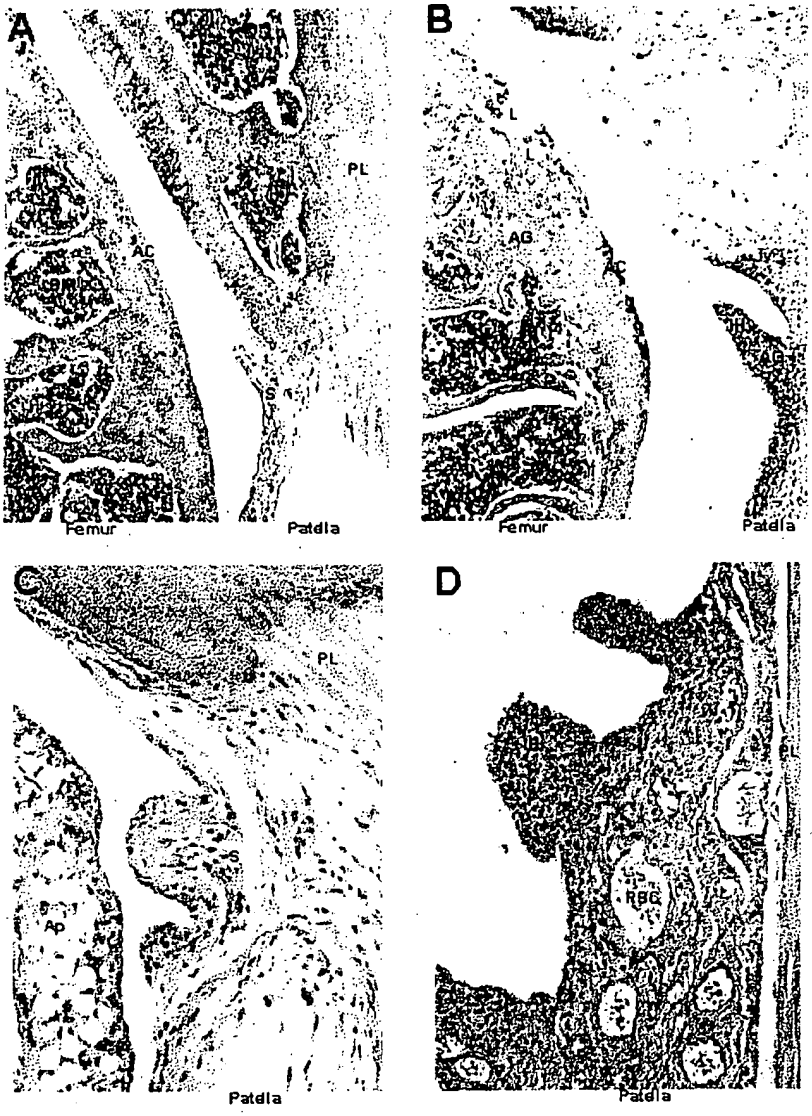
Wild Type (5 mos)

Line 6-1 (5 mos)

Line 99-1 (5 mos)

Conclusions

- Two lines of mice expressing the human MMP13 (collagenase-3) in the articular cartilage have been successfully created
- IHC data showed: (i) expression of hMMP13 protein in the articular cartilage
  (ii) cleavage of type II collagen fibrils
  (iii) increased expression of type X collagen
- When hMMP13* gene is expressed, these mice display characteristics of OA within 5 months
- Transgenic OA symptoms include:
  1. slow onset as the mouse ages, etc.
  2. erosion of the cartilage surface
  3. lesions
  4. osteophyte formation
  5. synovial hyperplasia
  6. disorganization/loss of chrondrocytes
- The present data validates MMP13 as a potential target for the intervention of OA
- These mice will provide a platform for the evaluation of lead compounds by the Discovery Team



Longitudinal sections through the hind knee joints. (A.) Age match litter mate control and (B.) line 6 removed from Dox. (C.) synovium of an age matched litter mate control and (D.) synovium of line 6 removed from Dox. Abbreviations: L, lesion; AC, articular cartilage; AG, angiogenesis; IH, infiltration hyperplasia; BM, bone marrow; and PL, patella ligament.